



---

## Identification of an Attractin-Like Pheromone in the Mucus-Secreting Hypobranchial Gland of the Abalone *Haliotis asinina* Linnaeus

Author(s): Chitraporn Kuanpradit, Scott F. Cummins, Bernard M. Degnan, Prapee Sretarugsa, Peter J. Hanna, Prasert Sobhon and Jittipan Chavadej

Source: Journal of Shellfish Research, 29(3):699-704.

Published By: National Shellfisheries Association

DOI: <http://dx.doi.org/10.2983/035.029.0321>

URL: <http://www.bioone.org/doi/full/10.2983/035.029.0321>

---

BioOne ([www.bioone.org](http://www.bioone.org)) is a nonprofit, online aggregation of core research in the biological, ecological, and environmental sciences. BioOne provides a sustainable online platform for over 170 journals and books published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Web site, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at [www.bioone.org/page/terms\\_of\\_use](http://www.bioone.org/page/terms_of_use).

Usage of BioOne content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

## IDENTIFICATION OF AN ATTRACTIN-LIKE PHEROMONE IN THE MUCUS-SECRETING HYPOBRANCHIAL GLAND OF THE ABALONE *HALIOTIS ASININA* LINNAEUS

CHITRAPORN KUANPRADIT,<sup>1,2</sup> SCOTT F. CUMMINS,<sup>2</sup> BERNARD M. DEGNAN,<sup>2</sup>  
PRAPEE SRETARUGSA,<sup>1</sup> PETER J. HANNA,<sup>1,3</sup> PRASERT SOBHON<sup>1</sup> AND  
JITTIPAN CHAVADEJ<sup>1\*</sup>

<sup>1</sup>Department of Anatomy, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand; <sup>2</sup>School of Biological Sciences, University of Queensland, Brisbane QLD 4072, Australia;

<sup>3</sup>Faculty of Science and Technology, Deakin University, Geelong, VIC 3217, Australia

**ABSTRACT** Pheromones are chemicals used to communicate between animals of the same species, and are thought to be used by most marine animals. With limited vision, abalone primarily sense their world chemically, and pheromones may play an important role in settlement, attraction, recognition, alarm, and reproduction. Despite this, there has been no detailed investigation into pheromone substances, both in their precise biochemical nature or pheromonal function. In this study, we investigated the presence of pheromonelike substances from the hypobranchial gland of the abalone *Haliotis asinina* using bioassays, immunohistochemistry, Western blotting, and reverse-phase high-performance liquid chromatography (RP-HPLC). The hypobranchial gland of many prosobranchial marine molluscs has been classified as a sex auxiliary gland releasing unknown substances during spawning. In our study, cephalic tentacle assays demonstrated that the cell extracts of the hypobranchial gland contain chemical cues that are sensed by conspecifics. An antibody against the sea slug “attractin” pheromone was used as a probe to localize a similar protein in the mucin-secreting cells of the epithelial lining the hypobranchial gland of both male and female abalone. The approximate molecular weight of this abalone attractin-like protein is 30 kDa in both males and females. Fractionation of hypobranchial gland extracts by C5 RP-HPLC could not selectively purify this protein, and no sex-specific differences were observed. We predict that the attractin-like protein could be one of a number of important proteins involved in maturation, aggregation, and/or spawning behavior of abalone. In future research, additional hypobranchial gland components will be tested further for these types of behavior.

**KEY WORDS:** abalone, pheromone, attractin, hypobranchial gland, mucus, *Haliotis asinina*

### INTRODUCTION

The prosobranch marine mollusc *Haliotis asinina* is an economically valuable aquaculture species in Thailand and other tropical countries. As such, many studies have been directed toward increasing its production in aquaculture (Fermin & Buen 2002, Klinbunga et al. 2003). In the wild, this species is benthic and typically prefers the protection of rocks and reef environments. Similar to other abalone species, they are solitary animals, but are known to aggregate during the spawning season to broadcast spawn their gametes (Shepherd 1986). This behavior is essential to increase the chances of successful gamete fertilization. Such attraction behaviors are likely to involve chemical triggers, the most ancient form of sensory communication (Wyatt 2003).

From observations of culturing *H. asinina* at the Coastal Aquaculture Development Center, Klong Wan, Prachuabkirikhan, Thailand, one of the major problems is that gametes are released in an asynchronized fashion. In many cases, only males release their gametes, a phenomenon resulting in a low fertilization success rate, because sperm remain viable for only 48 h post-spawning. Alternatively, in the case when only females spawn, their eggs will survive only 24 h postspawning, leading to the necessity to kill male broodstock to obtain testicular sperm for *in vitro* fertilization. These scenarios clearly indicate the requirement for key substances for mate recognition in preparation for synchronized release of gametes.

Pheromones are chemicals used to communicate between animals of the same species and are thought to be used by most marine animals. Based on experimental attraction assays performed on the hermaphroditic opisthobranch mollusc *Aplysia*,

chemical substances are released and detected by others of the same species, resulting in mate attraction and mating (Susswein et al. 1984, Painter et al. 1991, Audesirk 1997, Painter et al. 1998, Susswein & Nagle 2004, Cummins et al. 2005). The major water-borne pheromone involved is attractin, which is produced in the albumin gland and released at egg laying (Painter et al. 1998). Attractin is a 58-residue *N*-glycosylated protein with 3 intramolecular disulfide bonds; the precursor protein contains a single copy of attractin (Fan et al. 1997, Painter et al. 1998, Cummins et al. 2005). The responses of attractin in *Aplysia* include (1) locomotion to the arm of a T maze containing attractin, (2) an increase in respiratory pumping; and (3) a reduced latency to mating and an increase in the number of animals mating as hermaphrodites, which may result from attractin stimulating both animals to mate as males (Painter et al. 1989).

With limited vision, abalone primarily sense their world chemically, and pheromones most likely play important roles in settlement, attraction, recognition, and reproduction. For abalone, environmental cues are detected by 2 main olfactory appendages: the cephalic and epipodial tentacles (Wanichanon et al. 2004b). Our scanning electron micrograph and histological analysis of these appendages clearly defined their sensory role, in which they display numerous cilia-containing cells (Wanichanon et al. 2004b). Searching chemosensory behavior appears to be initiated when cephalic tentacles extend and retract, whereas the epipodial tentacles are likely distance chemosensory sensors. The activity of these olfactory organs are most apparent during the critical period leading up to synchronous spawning, when their sensory cephalic tentacles and epipodia become highly active, as if sensing for the appropriate chemical cue to trigger aggregation and spawning.

\*Corresponding author. E-mail: scjcv@mahidol.ac.th

In prosobranch gastropods, numerous organs could potentially be the source of pheromones, most likely derived from sex auxiliary glands, or glands attached to the reproductive tract, which release chemical substances related to reproductive function (Adiyodi & Adiyodi 1988). In *H. asinina*, the hypobranchial gland is of particular interest. It is located at the dorsal surface of the mantle cavity with a yellow pectinated ridge. Posteriorly, one fifth of this gland is attached to the rectum, which is located near the sperm and egg ducts through which gametes are released during spawning. The histological components of this gland have been described, of which the epithelium has 3 major components: the supporting cells, the sensory cells, and various mucus cell types (Wanichanon et al. 2004a). The variety of mucus-secreting cells indicates that the principle function of this gland in abalone is directed to mucus secretion (Wanichanon et al. 2004a). Mucus contains a settlement-inducing hormone,  $\gamma$ -aminobutyric acid (GABA) (Laimek et al. 2008), but there has been little evidence for it being a source of pheromone.

In this study we hypothesized that the function of the abalone hypobranchial gland might include pheromone production and release required for animal attraction and aggregation. However, to date there has been no reported evidence implicating a reproduction-related function of this gland in abalone. Therefore, the current study investigated the potential role of pheromone substances within the hypobranchial gland of *H. asinina*.

## MATERIALS AND METHODS

### Protein Preparation from Abalone Hypobranchial Gland

Adult male and female *H. asinina* (10 each), with maturing gonads, were anesthetized by immersion in an ice bath for 15 min. The hypobranchial glands were rapidly dissected, then washed in 0.1 M phosphate-buffered saline (PBS; pH, 7.4) before being placed into 1.5-mL microfuge tubes for liquid nitrogen freezing and storage at  $-80^{\circ}\text{C}$ . Proteins were extracted by homogenizing pooled hypobranchial glands at  $4^{\circ}\text{C}$  in 0.1% tri fluoroacetic acid (TFA) using a Polytron homogenizer (Brinkmann Instruments,

Canada), followed by sonication. The extracts were centrifuged (20,000g for 30 min at  $4^{\circ}\text{C}$ ) and lyophilized. These crude extracts were used for cephalic tentacle contraction assays and Western blot analysis. Some of the crude extract was further size fractionated through a 10-kDa NanoSepCentrifugal protein cutoff device filter (PALL Life Sciences, Port Washington, NY), using the manufacturer's instructions. The flow-through ( $<10$  kDa) and concentrated ( $>10$  kDa) proteins were collected for cephalic tentacle assays and reverse-phase high-performance liquid chromatography (RP-HPLC).

### Animal Preparation and Cephalic Tentacle Contraction Bioassay

A schematic representation of the experimental setup is shown in Figure 1A. For practical reasons, experiments were conducted using nonreproductively active adult (approximately 2- y-old) male and female abalone;  $n = 4$  for each sex to assess hypobranchial gland protein extract effects on conspecific cephalic tentacles. Morphological sex differences were not apparent because of the time of season. Animals were anesthetized in isotonic  $\text{MgCl}_2$  prior to head removal that left the cerebral and pleuropedal ganglia intact (adapted from Cummins et al. (2008)). Then, heads were gently transferred to a rectangular tank ( $20 \times 10 \times 10$  cm) containing 150 mL filtered artificial sea water (ASW; salinity, 32 ppt) at room temperature. The cephalic tentacles were monitored for a distinct movement (positive), and until twitching stopped, using a protractor in the background as a reference. The hypobranchial gland total protein extracts and size-fractionated samples were adjusted to  $10 \mu\text{g/mL}$  in filtered ASW. For stimulus application,  $50 \mu\text{L}$  solution was added by pipette to within 0.5–1 cm of the cephalic tentacles, which were then monitored for activity by video over a 30-sec period. The preparations were tested randomly and included male hypobranchial gland total extract ( $n = 5$ ), female hypobranchial gland total extract ( $n = 5$ ), male hypobranchial gland extract at more than 10 kDa ( $n = 3$ ) and less than 10 kDa ( $n = 3$ ), and female hypobranchial gland extract at more than 10 kDa ( $n = 3$ ) and less than 10 kDa ( $n = 3$ ). ASW was used as a negative vehicle control ( $n = 22$ ), alternated

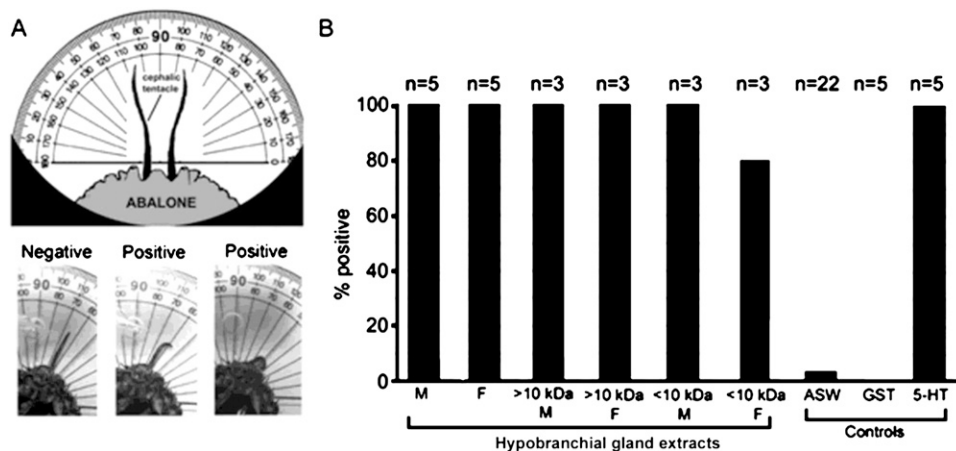


Figure 1. Organ bath bioassay for *Haliotis asinina* cephalic tentacle contraction. (A) Schematic representation of contraction assay experiment. Negative and positive responses to stimuli are shown. (B) Graph showing the effects of hypobranchial gland extracts on cephalic tentacles. Responses (contraction) of cephalic tentacles after stimulation with hypobranchial gland extracts of male (M) hypobranchial gland, female (F) hypobranchial gland, male hypobranchial gland ( $>10$  kDa), female hypobranchial gland ( $>10$  kDa), male hypobranchial gland ( $<10$  kDa), and female hypobranchial gland ( $<10$  kDa). Compared with the negative controls (ASW and GST), the numbers of cephalic tentacle contraction were significantly different ( $P < 0.05$ ), but not in comparison with the 5-HT positive control. 5-HT, 5-hydroxytryptamine; ASW, artificial seawater; GST, glutathione S-transferase.

with every test conditions; glutathione S-transferase (GST) 10 µg/mL was used as a nonspecific control ( $n = 5$ ), and the neurotransmitter 5-hydroxytryptamine (5-HT) serotonin 10 µg/mL was used as positive control ( $n = 5$ ). Between different test conditions the tank ASW was changed and tentacles were again monitored, typically about 5 min, before the next test. Statistical significance was assessed using a chi-square test.

#### *Specimen Preparation for Immunohistochemistry*

Five adult male and 5 adult female *H. asinina* (1 to 1.5-y-old and sexually mature) were obtained from the Coastal Aquaculture Research and Development Center, Department of Fisheries, Prachuabkirikhan Province, Thailand. Sex classification was achieved by inspection of gonad color (male gonad is an orange-cream color whereas female gonad is dark green). Animals were anesthetized in a 1:1 mixture of 7.5% MgCl<sub>2</sub> and seawater for 5 min before dissection. The hypobranchial glands were dissected and immediately fixed in Bouin's fixative for 24 h at 4°C. The fixed specimens were then dehydrated by an increasing series of ethanol changes and embedded in paraffin blocks. The paraffin blocks were sectioned at 5 µm and placed on slides precoated with (3-aminopropyl) triethoxy-silane for immunostaining.

#### *Immunohistochemistry*

To examine the expression of an attractin-like protein within abalone hypobranchial glands, sections of paraffin-embedded glands were cut, then deparaffinized in xylene, and rehydrated, and endogenous peroxidase activity was inactivated by treatment with 1% lithium carbonate in 70% ethanol for 15 min, followed by 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min, and washed in PBS containing 0.1% Tween 20 (PBST). Nonspecific binding was blocked by incubating sections in 1% normal goat serum and 5% bovine serum albumin in PBST, at 37°C for 1 h. Sections were then incubated in primary antibody against an *Aplysia californica* attractin pheromone (Cummins et al. 2004), diluted 1:500 in 1% bovine serum albumin at 37°C for 2 h in a moist chamber. Sections were washed 3 times for 10 min each in PBST to remove unbound antibody, then incubated in goat antirabbit immunoglobulin fluorescein isothiocyanate, diluted 1:10, for 1 h. Sections were washed 3 times and counterstained in the nuclear stain 4'-6-diamidino-2-phenylindole solution. Pictures were taken with a LSM5 PASCAL Laser confocal scanning microscope, after which the sections were counterstained with hematoxylin, washed in water, dehydrated, and mounted with Permount solution. For controls, sections were incubated in preimmune rabbit serum in blocking solution instead of primary antibody.

#### *Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blot Analysis*

Crude extracts were analyzed using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 10-µg samples from hypobranchial glands were loaded and separated by electrophoresis in 2 similar gels, one of which was stained with Coomassie Blue 250. Transfer of proteins from the other gel onto 0.45-µm-diameter polyvinylidene fluoride membrane was performed as detailed in Akalal et al. (2003). The membranes were washed with 0.1 M PBST before blocking with 5% skim milk in 0.1 M PBST at room temperature for 1 h. They

were incubated with attractin antiserum (1:1,000 dilution; 1 mg affinity-purified antibody/mL) for 8 h at 4°C, then washed 3 times in PBST for 5 min each, at room temperature. A secondary antibody, goat antirabbit immunoglobulin G HRP-conjugated diluted in PBST (1:10,000) was added and the membranes incubated at room temperature for 30 min. They were washed 3 times; antibody binding was detected using an enhanced chemiluminescence kit (Pierce) according to the manufacturer's instructions. For a negative control, the primary antiserum was replaced with preimmune serum.

#### *Reverse-Phase High-Performance Liquid Chromatography and Matrix-Assisted Laser Desorption Ionization Mass Spectrometry*

Hypobranchial gland extracts (male and female, >10 kDa) were purified by analytical Discovery BIO Wide Pore C5 HPLC (4.6-mm × 15-cm column, 5 µm) SUPELCO using a 2-step linear gradient (0–10% CH<sub>3</sub>CN/0.1% TFA in 5 min; 10–70% CH<sub>3</sub>CN/0.1% TFA in 65 min). The eluted fractions were monitored at 210 nm and 280 nm ultraviolet detection. Fractions (1 min) were lyophilized, and each fraction was analyzed by immunoassay as described previously (Western blot analysis). For matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), samples were added to MALDI matrix solution (20 mg/mL sinapinic acid in 50% CH<sub>3</sub>CN/0.1% TFA) and dried onto plates at ambient temperature until used for mass spectrometric analysis. MALDI-TOF mass spectrometry and calibration procedures were the same as described earlier, except that final spectra were recorded within a mass range from  $m/z$  500 to  $m/z$  12,000.

## RESULTS

#### *Cephalic Tentacle Contraction in the Presence of Hypobranchial Gland Extracts*

The summary of the results of responses after stimulation with hypobranchial gland extract or control preparations is shown in Table 1. Under control conditions, in which the tentacles were presented with ASW ( $n = 22$ ), no significant response was observed, indicating that mechanical stimulation was not a factor (Fig. 1B). The unrelated compound GST (used as a nonspecific control), caused no response ( $n = 5$ ), whereas the positive control using the neurotransmitter 5-HT, stimulated strong positive contractions ( $n = 5$ ). When cephalic tentacles were presented with any of the hypobranchial gland extract preparations, including male and female hypobranchial gland total extract ( $n = 5$  each), as well as size-fractionated more than 10-kDa and less than 10-kDa solutions ( $n = 3$  each), a significant positive response was observed ( $P < 0.03$ ; Fig. 1B). In each case, contraction was observed within 30 sec of presentation.

#### *Immunolocalization*

The presence of an attractin-like protein in *H. asinina* was established by immunofluorescence localization. Figure 2A and B shows the general cytological features of the abalone hypobranchial gland, including epithelia and mucus-secreting cells. The attractin-specific antibody localized a similar molecule within both male and female hypobranchial gland (female, Fig. 2C, D; male, Fig. 2E, F). Immunopositive material was located in mucus-secreting cells embedded in the epithelial



TABLE 1.  
Cephalic tentacle contraction assay.

Test Solution	ASW Vehicle Control 1	GST Nonspecific Control 2	5-HT* Control 3	Whole Male HBG Extract	Whole Female HBG Extract	>10 kDa Male HBG Fraction	>10 kDa Female HBG Fraction	<10 kDa Male HBG Fraction	<10 kDa Female HBG Fraction
No. of tentacle movements	1 (n = 22)†	0 (n = 5)	5 (n = 5)	5 (n = 5)	5 (n = 5)	3 (n = 3)	3 (n = 3)	3 (n = 3)	2 (n = 3)

\* Serotonin neurotransmitter.

† Number tested.

ASW, artificial seawater; GST, glutathione S-transferase; HBG, hypobranchial gland.

regions, as well as in secreted mucus in the external environment. For negative controls in which no primary antibody was used, all immunoreactivity was absent (data not shown).

#### SDS-PAGE and Western Blotting

Proteins extracted from hypobranchial glands were separated by 12% SDS-PAGE, and visualized by Coomassie Blue, as shown in Figure 3. Western blot analysis (of the separated proteins), performed using antiattractin, reacted with an approximately 30-kDa protein within extracts derived from both male

and female hypobranchial glands (Fig. 3). No immunoreactive band was observed at the size of *Aplysia* attractin (approximately 6.3 kDa) using the conditions described previously.

#### RP-HPLC Purification and MALDI-MS Analysis of the Hypobranchial Gland

We found that hypobranchial gland extracts could induce a cephalic tentacle contraction response in conspecifics, and that Western blotting predicted an attractin-like protein of more than 10 kDa within secretory cells of this gland. Therefore, we wanted to identify precisely the individual biomolecules within the hypobranchial gland that could induce these cephalic responses. For this, we used protein extracts prepared as described for cephalic tentacle bath assay (>10 kDa size-fractionated samples), then further processed them by semipreparative RP-HPLC. Representative RP-HPLC elution profiles of male and female hypobranchial gland protein are shown in Figure 4A and B. Immunoblot analysis of individual fractions (1 min) using the antiattractin could not definitively locate the attractin-like protein from any of the RP-HPLC preparations (data not shown). The patterns of peak elution in male and female HBG appear similar, but there were some obvious differences within fractions 43–44. Subsequent mass spectrometry analysis of the major absorbance peaks within these fractions, however, showed that there is no distinct difference between sexes. For example, MALDI-MS showed m/z charge peaks of 4,868.4 and 4,891.9 in fraction 43–44 for both male and female (Fig. 4C).

#### DISCUSSION

The complex biochemical nature of hypobranchial gland extracts likely reflects the importance of this tissue in biological homeostasis, in the production of mucus, soluble proteins, carbohydrates, secondary metabolites (Naegel et al. 2006), and possibly molecules involved in animal communication. Here, we hypothesized that there could be molecules released, even sex-specific cues, that might ensure females are preferentially attracted to conspecific males, or vice versa.

As a preliminary study to the eventual characterization of specific pheromonal biomolecules, we assayed animal response to hypobranchial gland extracts. For abalone, environmental cues are detected by 2 main olfactory appendages—the cephalic and epipodial tentacles (Wanichanon et al. 2004b). For practical purposes we chose to focus on the cephalic tentacles, and contraction assays were used to assess chemosensory stimulants present within hypobranchial gland extracts. Contraction correlates with sensory cell detection, likely leading to a hormonal

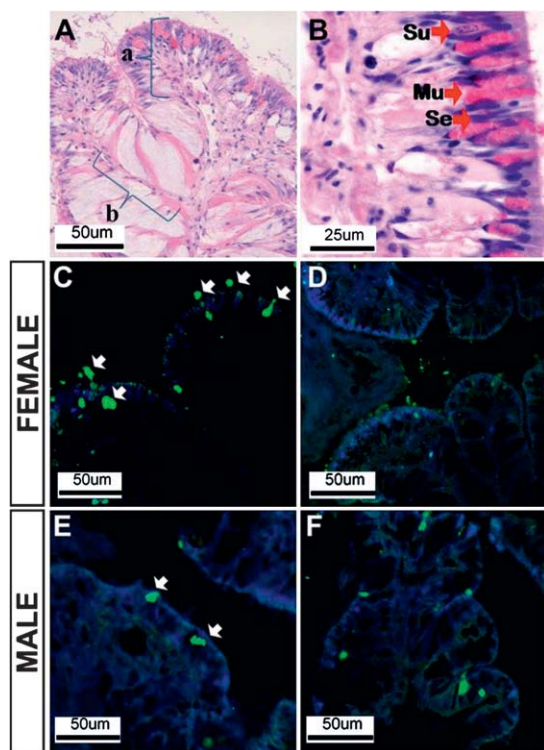


Figure 2. Identification of an attractin-like protein in the hypobranchial gland of *Haliotis asinina*. Immunofluorescence was performed using a primary antibody that detects *Aplysia* attractin. (A, B) Low-power (A) and high-power (B) hematoxylin-eosin staining of hypobranchial gland sections. (C, D) Immunopositive staining (arrows) was detected within mucus-secreting cells of the female hypobranchial gland. (E, F) Immunopositive staining (arrows) was detected within the mucus-secreting cells of the male hypobranchial gland. Sections were counterstained with 4'-6-diamidino-2-phenylindole (blue). a, apical tissue; b, basal tissue; Mu, mucus-secreting cell; Se, sensory cell; Su, supporting cell.

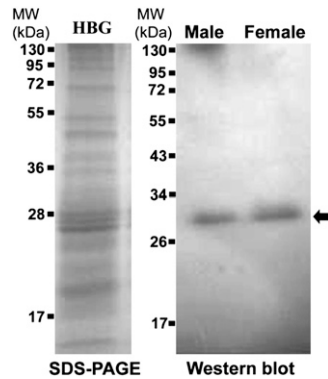


Figure 3. Western blot analysis of crude protein extracts of *Haliotis asinina* hypobranchial glands (HBG). Proteins extracted from male and female hypobranchial glands were separated by 12% SDS-PAGE and then stained with Coomassie Blue R-250. They showed a complex pattern of bands (combined male and female). Proteins were also transferred to polyvinylidene fluoride for Western blot analysis and probed using an antiattractin antibody. In both male and female extracts, an attractin-like protein was identified as indicated by an immunoreactive band at approximately 30 kDa (arrow). Female, female HBG; Male, male HBG; MW, molecular weight markers.

followed by a behavior response. We discovered that sensory chemoreceptors present on cephalic tentacles appear to be activated by whole hypobranchial gland prepared extracts, as well as size-fractionated samples.

The precise function of hypobranchial gland biomolecules has only been established in a few cases in some molluscs (Gribble 1999). Some of these biomolecules have been hypothesized to affect membrane transport ion channels. For example, investigations have demonstrated that mucus secretions from this gland likely contain bioactive molecules, such as novel potassium channel antagonists found in *Calliostoma canaliculatum* (Kelley et al. 2003). Also, a study by Roseghini et al. (1996) revealed that hypobranchial glands contain choline esters and biogenic amines that display potent neuromuscular blocking actions in vertebrate and invertebrate species, as well as potent nicotinic actions. Closely related to the current study, Laimek et al. (2008) found that abalone mucus, some of which is expected to be derived from the hypobranchial gland, contained GABA, a chief inhibitory neurotransmitter that influences the induction of larval settlement. In that study, GABA could have also functioned as a chemosensory attractant for larvae, but unknown pheromones were also considered.

In our study, we have shown by immunolocalization that the mucus-secreting cells of the abalone hypobranchial gland contain an attractin-like protein that could be similar to that described in *Aplysia* by Painter et al. (1998). The attractin-like protein, however, is not sex specific and is present within both male and female glands. Besides similarity, as demonstrated by cross-reactivity to the *A. californica* antibody, the abalone equivalent is likely to be quite different. Indeed, based on SDS-PAGE and Western blotting, the abalone attractin is approximately 30 kDa in size within both males and females. This predicted size is in contrast to the relative small size of *A. californica* attractin: 6.3 kDa (Painter et al. 1998). These data suggest that the abalone attractin is larger, but likely contains conserved regions, similar to the second alpha helix that encompasses the heptapeptide sequence Ile<sub>30</sub>-Glu<sub>31</sub>-Glu<sub>32</sub>-Cys<sub>33</sub>-Lys<sub>34</sub>-Thr<sub>35</sub>-Ser<sub>36</sub> (IEECKTS)

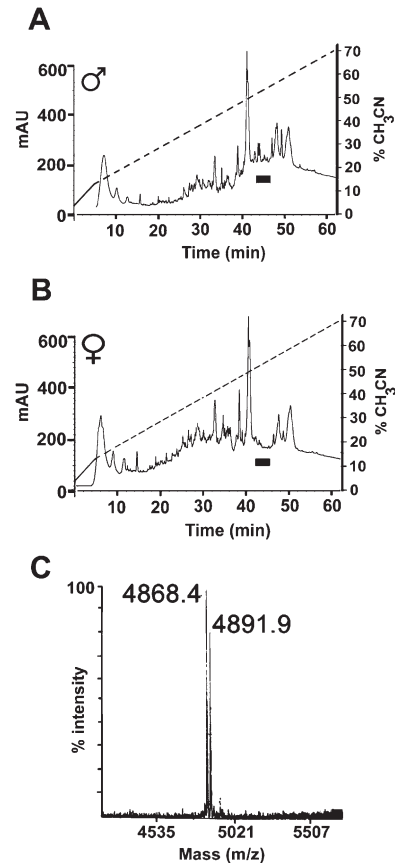


Figure 4. RP-HPLC and MALDI-MS of size-fractionated *Haliotis asinina* hypobranchial gland extracts. (A, B) Comparative RP-HPLC profile showing C5 column separation of male (A) and female (B) hypobranchial gland proteins of more than 10 kDa. Solid bars represent peak fractions assayed by MALDI-MS. (C) MALDI-MS analysis showing a major m/z peak of 4,868, mass-to-charge ratio.

of *Aplysia* attractin. Peptide sequences of attractin in the aplysiid species *A. californica*, *A. brasiliana*, *A. fasciata*, *A. vaccaria*, and *A. depilans* (Painter et al. 2004), show conservation of 6 cysteines, 3 charged residues (Asp<sub>5</sub>, Asp/Glu<sub>22</sub>, and Glu<sub>39</sub>), and the sequence (IEECKTS). Although this highly conserved heptapeptide sequence is important for biological activity (Cummins et al. 2004), its presence has been understudied outside *Aplysia*. Our failures to purify the abalone attractin molecule using RP-HPLC with a C5 (or C18 RP-HPLC) column, suggest that despite some structural homology to *Aplysia* attractin, it does not bind to these columns. Alternative purification methods will be necessary to elucidate fully the biochemical nature of this molecule.

Other observations show that this attractin-like protein is present in other abalone tissues, including the foot and gills (data not shown). These observations at the cellular level reveal that it is found within secretory-type cells of these tissues and, like the hypobranchial gland, could release this protein into the external environment via secretion. Extensive analysis of RP-HPLC extracts by MALDI-MS could not identify a unique sex-specific peptide/protein, although peaks within the fractions 43–44 min contained molecules that appeared to be only present in male gland extracts. However, further analysis by MALDI-MS demonstrated that these fractions contained proteins of mass size 4,868 m/z, and were present in both sexes. Our next step will

be to define the precise stimulatory chemical within RP-HPLC fraction peaks that can be further characterized.

There is abundant evidence for the role of the hypobranchial gland in transport and expulsion of debris (Westley et al. 2006), as well as functioning in the *de novo* biosynthesis and storage of secondary metabolites (Westley & Benkendorff 2008). However, we demonstrate here for the first time that the hypobranchial gland could play a role in animal signaling, because abalone respond to extracts of conspecific hypobranchial glands. Further RP-HPLC fractionation of active extracts will ultimately help define the precise molecules involved in this response. Immunolocalization, using an antibody specific to *Aplysia* attractin, showed that it is possible that an abalone attractin-like protein could be released from the hypobranchial gland and detected by conspecific abalone. Further characterization, and synthesis, of this attractin-like

protein is now being carried out to assess its role in abalone sex attraction, aggregation, and synchronous spawning behavior.

#### ACKNOWLEDGMENTS

This work was supported by the Thailand Research Fund (TRF), the Commission on Higher Education and Mahidol University (P. S. and J. C.), a TRF- RGJ PhD scholarship (C. K.), and a Hermon Slade Foundation award (S. C.). We thank W. Suphamongmee, P. Stewart, N. Soonklang, and P. Nuurai for technical and experimental assistance; T. Poomtong (Coastal Aquaculture Development Center, Klong Wan, Prachuabkirikhan, Thailand) for providing abalone culture facilities; P. S. York for assistance with abalone culture in Australia; and G. T. Nagle for kindly donating the attractin antibody.

#### LITERATURE CITED

- Adiyodi, K. G. & R. G. Adiyodi. 1988. Reproductive biology of invertebrates: accessory sex glands. In: Reproductive biology of invertebrates, vol. III. John Wiley & Sons. pp. 176–178.
- Akalal, D. B., J. E. Bottenstein, S. H. Lee, J. H. Han, D. J. Chang, B. K. Kaang & G. T. Nagle. 2003. *Aplysia* mollusk-derived growth factor is a mitogen with adenosine deaminase activity and is expressed in the developing central nervous system. *Brain Res Mol Brain Res* 117:228–236.
- Audesirk, T. E. 1979. Field-study of growth and reproduction in *Aplysia californica*. *Biol. Bull.* 157:407–421.
- Cummins, S. F., B. M. Degnan & G. T. Nagle. 2008. Characterization of *Aplysia* Alb-1, a candidate water-borne protein pheromone released during egg laying. *Peptides* 29:152–161.
- Cummins, S. F., A. E. Nichols, K. Rajarathnam & G. T. Nagle. 2004. A conserved heptapeptide sequence in the waterborne attractin pheromone stimulates mate attraction in *Aplysia*. *Peptides* 25:185–189.
- Cummins, S. F., A. E. Nichols, C. H. Schein & G. T. Nagle. 2005. Newly identified water-borne protein pheromones interact with attractin to stimulate mate attraction in *Aplysia*. *Peptides* 27:597–606.
- Fan, X., W. Bo, G. T. Nagle & S. D. Painter. 1997. Short communication molecular cloning of a cDNA encoding a potential water-borne pheromonal attractant released during *Aplysia* egg laying. *Brain Res. Mol. Brain Res.* 48:167–170.
- Fermin, A. C. & S. M. Buen. 2002. Grow-out culture of tropical abalone, *Haliotis asinina* (Linnaeus) in suspended mesh cages with different shelter surface areas. *Aquacult. Int.* 9:499–508.
- Gribble, G. W. 1999. The diversity of naturally occurring organobromine compounds. *Chem. Soc. Rev.* 28:335–346.
- Kelley, W. P., A. M. Wolters, J. T. Sack, R. A. Jockusch, J. C. Jurchen, E. R. Williams, J. V. Sweedler & W. F. Gilly. 2003. Characterization of a novel gastropod toxin (6-bromo-2-mercaptotryptamine) that inhibits shaker K channel activity. *J. Biol. Chem.* 278:34934–34942.
- Klinbunga, S., P. Pripue, N. Khamnamtong, N. Puanglarp, A. Tassanakajon, P. Jarayabhand, I. Hirano, T. Aoki & P. Menasveta. 2003. Genetic diversity and molecular markers of the tropical abalone (*Haliotis asinina*) in Thailand. *Mar. Biotechnol.* 5:505–517.
- Laimek, P., S. Clark, M. Stewart, F. Pfeffer, C. Wanichanon, P. Hanna & P. Sobhon. 2008. The presence of GABA in gastropod mucus and its role in inducing larval settlement. *J. Exp. Mar. Biol. Ecol.* 354:182–191.
- Ludwig, C., A. Naegel & C. A. Aguilar-Cruz. The hypobranchial gland from the purple snail *Plicopurpura pansa* (Gould 1853) (Prosobranchia: Muricidae). *J. Shellfish Res.* 25:391–394.
- Painter, S. D., M. G. Chong, M. A. Wong, A. Gray, J. Cormier & G. T. Nagle. 1991. Relative contributions of the egg layer and egg cordons to pheromonal attraction and the induction of mating and egg-laying activity in *Aplysia*. *Biol. Bull.* 181:81–94.
- Painter, S. D., B. Clough, R. W. Garden, J. V. Sweedler & G. T. Nagle. 1998. Characterization of *Aplysia* attractin, the first water-borne peptide pheromone in invertebrates. *Biol. Bull.* 194:120–131.
- Painter, S. D., S. F. Cummins, A. E. Nichols, D. B. G. Akalal, C. H. Schein, W. Braun, J. S. Smith, A. J. Susswein, M. Levy, P. A. C. M. de Boer, A. ter Maat, M. W. Miller, C. Scanlan, R. M. Milberg, J. V. Sweedler & G. T. Nagle. 2004. Structural and functional analysis of *Aplysia* attractins, a family of water-borne protein pheromones with interspecific attractiveness. *Proc. Natl. Acad. Sci. USA* 101:6929–6933.
- Painter, S. D., A. R. Gustavson, V. K. Kalman, G. T. Nagle & J. E. Blankenship. 1989. Induction of copulatory behavior in *Aplysia*: atrial gland factors mimic the excitatory effects of freshly deposited egg cordons. *Behav. Neural Biol.* 51:222–236.
- Roseghini, M., C. Severini, G. F. Erspamer & V. Erspamer. 1996. Choline esters and biogenic amines in the hypobranchial gland of 55 molluscan species of the neogastropod Muricoidea superfamily. *Toxicon* 34:33–55.
- Shepherd, S. A. 1986. Studies on southern Australian abalone (genus *Haliotis*): VII. Aggregative behavior of *H. laevigata* in relation to spawning. *Mar. Biol.* 90:231–236.
- Susswein, A. J., S. Gev, Y. Achituv & S. Markovich. 1984. Behavioral patterns of *Aplysia fasciata* along the Mediterranean coast of Israel. *Behav. Neural Biol.* 41:7–22.
- Susswein, A. J. & G. T. Nagle. 2004. Peptide and protein pheromones in molluscs. *Peptides* 25:1523–1530.
- Wanichanon, C., P. Laimek, N. Chitchulanon, W. Suphamongmee, S. Apisawetakan, V. Linthong, P. Sretaruga, M. Kruatrachue, E. S. Upatham, T. Poomtong & P. Sobhon. 2004a. Sensory receptors on cephalic and epipodial tentacles of *Haliotis asinina* Linnaeus. *J. Shellfish Res.* 23:1097–1106.
- Wanichanon, C., P. Laimek, V. Linthong, P. Sretaruga, M. Kruatrachue, E. S. Upatham, T. Poomtong & P. Sobhon. 2004b. Histology of hypobranchial glands and gills of *Haliotis asinina* Linnaeus. *J. Shellfish Res.* 23:1107–1112.
- Westley, C. B. & K. Benkendorff. 2008. Sex-specific Tyrian purple genesis: precursor and pigment distribution in the reproductive system of the marine mollusc, *Dicathais orbita*. *Journal of Chemical Ecology* 34:44–56.
- Westley, C., K. Vine & K. Benkendorff. 2006. A proposed functional role for indole derivatives in reproduction and defense of the Muricidae Neogastropoda: Mollusca. In: L. Meijer, N. Guyard, A. L. Skaltsounis & G. Eisenbrand, editors. Indirubin, the red shade of indigo. France: Roscoff, Life in Progress Editions. pp. 31–44.
- Wyatt, T. D. 2003. Pheromone and animal behavior: communication by smell and taste. Cambridge: Cambridge University Press. pp. 1–6.